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A GroEL Homologue from Endosymbiotic Bacteria of the Whitefly *Bemisia tabaci* Is Implicated in the Circulative Transmission of Tomato Yellow Leaf Curl Virus

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Evidence for the involvement of a *Bemisia tabaci* GroEL homologue in the transmission of tomato yellow leaf curl geminivirus (TYLCV) is presented. A ~63-kDa protein was identified in *B. tabaci* whole-body extracts using an antiserum raised against aphid *Buchnera* GroEL. The GroEL homologue was immunolocalized to a coccoid-shaped whitefly endosymbiont. The 30 N-terminal amino acids of the whitefly GroEL homologue showed 80% homology with that from different aphid species and GroEL from *Escherichia coli*. Purified GroEL from *B. tabaci* exhibited ultrastructural similarities to that of the endosymbiont from aphids and *E. coli*. *In vitro* ligand assays showed that tomato yellow leaf curl virus (TYLCV) particles displayed a specific affinity for the *B. tabaci* 63-kDa GroEL homologue. Feeding whiteflies anti-*Buchnera* GroEL antiserum before the acquisition of virions reduced TYLCV transmission to tomato test plants by >80%. In the haemolymph of these whiteflies, TYLCV DNA was reduced to amounts below the threshold of detection by Southern blot hybridization. Active antibodies were recovered from the insect haemolymph suggesting that by complexing the GoEL homologue, the antibody disturbed interaction with TYLCV, leading to degradation of the virus. We propose that GroEL of *B. tabaci* protects the virus from destruction during its passage through the haemolymph.

INTRODUCTION

Tomato yellow leaf curl virus (TYLCV) is the name given to a complex of genetically different geminiviruses (family Geminiviridae, genus *Begomovirus*) affecting tomato cultures worldwide (Czosnek and Laterrot, 1997). TYLCV from the Middle East and Southwest Europe has a monopartite genome, whereas TYLCV from Thailand has a bipartite genome (Padidam *et al.,* 1995; Ribycki, 1994). TYLCV is transmitted by the whitefly *Bemisia tabaci* in a persistent circulative manner (Cohen and Nitzany, 1966; Rubinstein and Czosnek, 1997). Although products encoded by both genomic components are required for transmission of the bipartite African cassava mosaic virus (ACMV; family Geminiviridae, genus *Begomovirus*) by *B. tabaci*, the only viral gene product necessary for acquisition is the coat protein (Liu *et al.,* 1997). Swapping coat proteins (CP) between a nontransmissible geminivirus (*Abutilon* mosaic virus; AbMV) and a transmissible one (*Sida* golden mosaic virus; SiGMV) resulted in regain of transmissibility of the AbMV-SiGMV:CP chimera (Höfer *et al.,* 1997). Moreover, the CP is involved in the molecular recognition of the virus by its vector. Exchanging the CP gene of the whitefly-transmitted ACMV with that of leafhopper-transmitted beet curly top virus (BCTV; family Geminiviridae, genus *Curtovirus*) produced a leafhopper-transmissible ACMV-BCTV:CP chimera (Briddon *et al.,* 1990).

The pathway of the virus in the insect vector and the cellular and molecular processes underlying whitefly-mediated geminivirus transmission are poorly understood. It is believed that virus particles are ingested along with phloem sap of infected host plants through the stylets and enter the esophagus and filter chamber (Harris *et al.,* 1995). Most likely, as observed for luteoviruses in aphids, virions are transported through the gut into the haemocoel. Virions that reach the salivary glands are translocated into the salivary duct, from which they are excreted with the saliva during feeding (Gildow and Gray, 1993; Gray, 1997). Immunolocalization studies have suggested that the *B. tabaci* filter chamber and anterior portion of the midgut are possible sites involved in geminivirus transport from the gut lumen to the haemocoel (Hunter *et al.,* 1998).

It has recently been suggested that a 63-kDa GroEL homologue plays a role in the circulative transmission of luteoviruses. Viruses from the genera *Luteovirus*, *Polerovirus,* and *Enamovirus* display a specific but differential affinity for this protein, which is produced by the primary endosymbiont (a *Buchnera* sp.) of aphids (van den Heuvel *et al.,* 1994, 1997; Filichkin *et al.,* 1997). These gram-

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negative bacteria are harboured in the aphid's haemocoel by specialized polyploid cells, called mycetocytes (Buchner, 1965), and are distantly related to *Escherichia coli* (Unterman *et al.,* 1989). *Buchnera* GroEL homologues are found in most aphid taxa and are immunologically closely related. Structural characteristics of *Buchnera* GroEL are highly similar to those of GroEL of *E. coli,* and a >80% sequence identity is present in functionally significant regions (Filichkin *et al.,* 1997; Hogenhout *et al.,* 1998; Ohtaka *et al.,* 1992; van den Heuvel *et al.,* 1994, 1997). However, unlike *E. coli* GroEL, *Buchnera* GroEL is not restricted to the cytosol of the bacteria. It is also present extracellularly in the aphid's haemolymph (van den Heuvel *et al.,* 1994, 1997). A treatment of *Myzus persicae* larvae with antibiotics that interfered with the prokaryotic protein synthesis dramatically lowered the level of *Buchnera* GroEL in the haemolymph and resulted in inhibited transmissibility and loss of capsid integrity of potato leafroll virus (PLRV; van den Heuvel *et al.,* 1994). Collectively, these results suggest that the luteovirus–GroEL interaction is required to retard proteolytic breakdown in the haemolymph and is essential for virus retention in the aphid (van den Heuvel *et al.,* 1994, 1997).

Whiteflies, like aphids and most other homopterous insects, contain endosymbiotic microorganisms that are housed in mycetocytes (Buchner, 1965). Two morphologically distinct types of microorganisms are present in each mycetocyte. The predominant endosymbiont in *B. tabaci* B biotype (Costa *et al.,* 1995) is highly pleomorphic (P-type), constitutes a distinct lineage within the γ subdivision of *Proteobacteria*, and is unrelated to the aphid primary endosymbiont (Baumann *et al.,* 1993). The second type of *B. tabaci* endosymbionts is a coccoid bacterium that is found in lower numbers than the P-type and is closely related to the aphid endosymbiont (Baumann *et al.,* 1993; Clark *et al.,* 1992).

Here we show the involvement of whitefly endosymbionts in the circulative transmission of TYLCV by *B. tabaci*.

RESULTS

A *Buchnera* GroEL antibody labels preferentially the cytoplasm of *B. tabaci* coccoid endosymbionts

The *B. tabaci* population used for the localization studies contained two types of morphologically different bacterium-like organisms within their mycetocytes (Fig. 1). The predominant endosymbiont was highly pleomorphic (P-type) and constituted ~80% of the total endosymbiont population in a mycetocyte, as previously described for the *B. tabaci* B biotype (Costa *et al.,* 1995). The second type is coccoid in shape (C-type; Costa *et al.,* 1995), and two size classes can be distinguished. The major C-type endosymbiont is 0.8–1.0 μm wide and up to 4.5 μm in length (Fig. 1B); a less abundant one is 2–3 μm in diameter, which resembles morphologically the aphid primary endosymbiont (Fig. 1A). Within the whitefly mycetocytes, the C-type endosymbionts were found singly (Fig. 1A), in groups (not shown), and within vacuoles (Fig. 1B). In most cases, these vacuoles contained intact bacteria, apparently degrading bacteria, and varying amounts of amorphous electron-dense bodies. Immunogold labeling studies using the antibody to *Buchnera* GroEL revealed that gold label was clearly detected in association with the granular electron-dense material in the cytoplasm of the C-type endosymbionts. The electron-dense bodies in the vacuoles were heavily tagged as well. In contrast, the P-type bacteria were hardly labeled. The amount of gold particles found in their cytoplasm was only slightly higher than background labeling.

Amino acid sequence of the N-terminus of *B. tabaci* GroEL shows a high degree of homology with GroEL from *M. persicae* and *E. coli*

The aphid endosymbiotic bacteria (*Buchnera* sp.) synthesize large amounts of a GroEL homologue with subunits of 63 kDa, previously named symbionin (Ishikawa, 1982, 1984). We assessed whether the *B. tabaci* C-type endosymbionts harbor a similar protein. Fractions enriched in GroEL were obtained after subjecting homogenates of adult *B. tabaci* to ultracentrifugation through a sucrose gradient. Western blot analysis using an antiserum to *Buchnera* GroEL revealed a single ~63-kDa band corresponding to the GroEL subunit (Fig. 2A). The protein reacting with the antibody was isolated from a gel after SDS–PAGE, and the sequence of the 30 N-terminal amino acids was obtained by Edman degradation (Fig. 3). The *B. tabaci* protein showed 80% sequence identity with homologues from *M. persicae* (van den Heuvel *et al.,* 1994) and *E. coli* (Hemmingsen *et al.,* 1988). Amino acids at positions 3 (lysine), 12 (arginine), 18 (glycine), 25–26 (alanine–valine), and 29 (threonine), previously shown to be conserved in other GroEL/Hsp60 proteins (Hogenhout *et al.,* 1998), were also present in *B. tabaci* GroEL. Among the six changes compared with *Buchnera* sp. and *E. coli* GroEL (leucine at position 5, aspartic acid at position 10, lysine at position 13, lysine at position 17, isoleucine at position 21, and asparagine at position 24), only lysine at position 13 is not a substitution of the same amino acid subgroup. However, this basic substitution was found in chaperonin-60 of *Pseudomonas putida* (GenBank accession no. P48216) and *Chlamydia psittaci* (GenBank accession no. P15599); an acidic conversion was found in chaperonin-60 of several other species. Therefore, we assume that this position has not been conserved during evolution. Although it cannot be excluded that small amounts of protein from the pleomorphic bacteria, which immunoreacted with the *Buchnera* GroEL antiserum, have been copurified with the major 63-kDa product from the *B.*

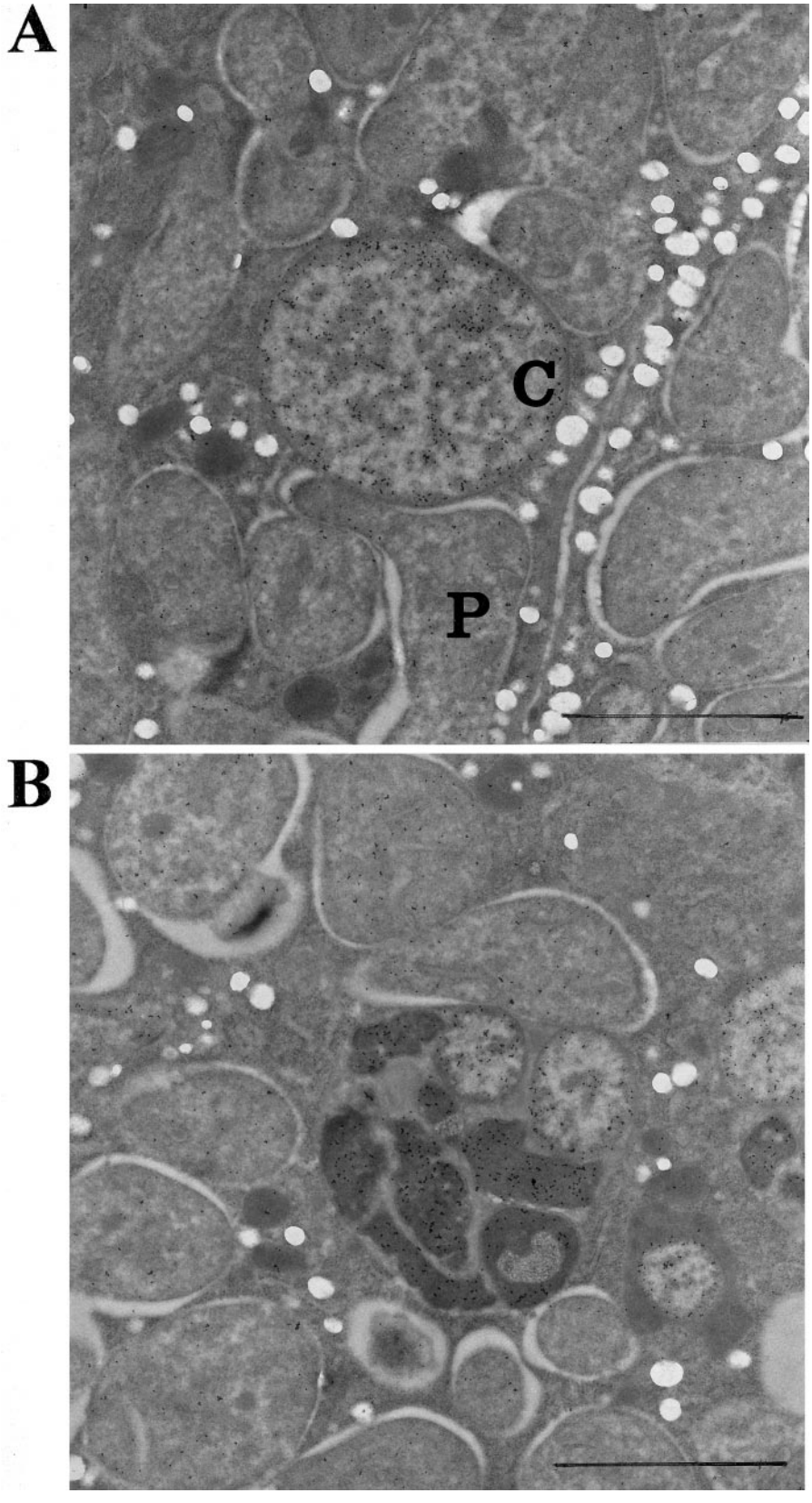

FIG. 1. Electron micrograph of *B. tabaci* mycetocytes showing immunogold labeling of the coccoid (C) endosymbionts of *B. tabaci* using an antiserum raised against *Buchnera* GroEL. P indicates pleomorphic endosymbionts. (A) C-type endosymbiont found singly. (B) C-type endosymbiont found in groups within a vacuole. Bar = 3 μm.

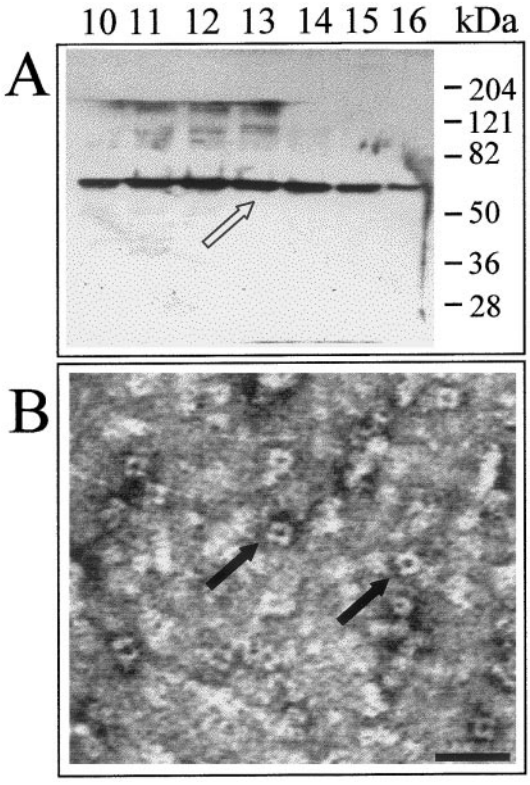

FIG. 2. Purification of *B. tabaci* GroEL homologue. (A) After centrifugation of insect extracts in a 10–50% linear sucrose gradient, fractions 10–16 were submitted to SDS–PAGE, and the gel was stained with Coomassie Brilliant Blue. An identical gel was electroblotted, and the GroEL homologue was identified (white arrow) with an antiserum raised against *M. persicae Buchnera* GroEL. The ~63-kDa protein that reacted with the antibody was extracted from the first gel and microsequenced (see Fig. 3); the molecular weight markers are indicated on the right. (B) Electron micrograph of the *B. tabaci* GroEL-enriched fraction 13 from the sucrose gradient, stained with 2% (w/v) uranyl acetate. Arrows indicate top views of GroEL. Bar = 50 nm.

tabaci coccoid endosymbiont, it did not interfere with the N-terminal sequence determination because the data were unambiguous.

Electron microscopic observation of the *B. tabaci* GroEL-containing fraction from the sucrose gradient clearly showed the multimeric nature of the protein (Fig. 2B). The observed GroEL complexes were cylindrical structures of average size and with a general architecture similar to those described for GroEL from *M. persicae, Rhopalosiphum padi,* and *Sitobion avenae* (Filichkin *et al.,* 1997; van den Heuvel *et al.,* 1997).

B. tabaci	AAKD**L** KFGN**D** AR**K**KM L**K**GVN **I**LA**N**A VKVTL
M. persicae	AAKD**V** KFGN**E** AR**I**KM L**R**GVN **V**LA**D**A VKVTL
E. coli	AAKD**V** KFGN**D** AR**V**KM L**R**GVN **V**LA**D**A VKVTL

FIG. 3. Sequence of the N-terminal 30 amino acids of GroEL from *B. tabaci*, *M. persicae*, and *E. coli*. Amino acids not shared by all three proteins are in bold.

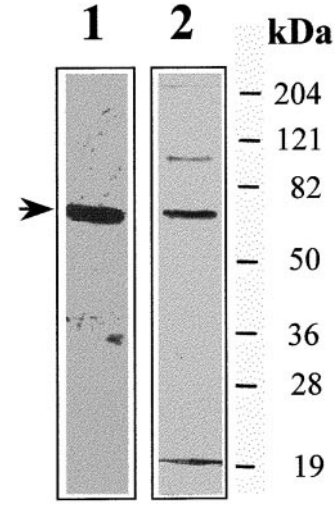

FIG. 4. Affinity of TYLCV for GroEL from *B. tabaci*. (1) Blot of an insect extract was incubated with TYLCV particles. TYLCV coat protein was detected with a monoclonal antibody. (2) *B. tabaci* proteins labeled with antibodies against *Buchnera* GroEL. The arrow points to the TYLCV coat protein that reacted with the ~63-kDa *B. tabaci* GroEL homologue.

TYLCV particles display affinity for the *B. tabaci* GroEL homologue

To determine whether the *B. tabaci* GroEL homologue has affinity to TYLCV virions, we have conducted a virus overlay assay similar to the one that was instrumental in demonstrating the interaction between PLRV and *Buchnera* GroEL. Whole-body homogenates of *B. tabaci* adults were subjected to SDS–PAGE and blotted onto a membrane. Part of this membrane was incubated with TYLCV virions, and immobilized virus particles were detected with antibodies against TYLCV coat protein. In this way, it was revealed that purified TYLCV bound to a single ~63-kDa protein (Fig. 4, lane 1). The other part of the membrane was incubated with an antibody raised against native *Buchnera* GroEL from *M. persicae* that was also used in the immunogold-labeling studies (see above). This antibody readily detected a band of ~63 kDa in the whole-body homogenate (Fig. 4, lane 2). In addition, a protein of ~90 kDa immunoreacted with the *Buchnera* GroEL antibodies. However, this protein failed to bind purified TYLCV in the overlay assay.

Antibodies to *Buchnera* GroEL interfere with the transmission of TYLCV by *B. tabaci*

To assess whether an interaction between the GroEL homologue from the C-type endosymbiont of *B. tabaci* and TYLCV occurs *in vivo*, antibodies to *Buchnera* GroEL were membrane-fed to whiteflies. The antibodies were offered to the whiteflies before and during virus acquisition from a sucrose solution. Control groups of whiteflies were prefed preimmune serum before virus acquisition. Antibody-treated and control insects were caged with tomato test plants for an inoculation-feeding period of 5

days. Three weeks later, these plants were tested for the presence of TYLCV DNA. The whiteflies that acquired the anti *Buchnera* GroEL were very poor vectors of the virus relative to those that were fed preimmune serum. Two experiments were carried out. In the first one, the control insects infected 27 of 29 plants, whereas the anti *Buchnera* GroEL-treated whiteflies infected only 1 of 30 plants. In the second experiment, the control group of whiteflies infected 12 of 26 plants, whereas the antibody-treated whiteflies infected only 2 of 26 plants. Therefore, treatment of whiteflies with anti-*Buchnera* GroEL antiserum before the acquisition of virions reduced TYLCV transmission to tomato test plants by >80%.

The fate of the ingested anti-*Buchnera* GroEL antibodies in the insect haemolymph was examined. To this end, haemolymph was sampled from 25 insects fed either anti-*Buchnera* GroEL or preimmune serum and was used as an antibody source in immunodetection experiments. In this way, we were able to detect GroEL in homogenates of *E. coli* (Fig. 5, lane 1), demonstrating that the haemolymph contained biologically active antibodies (Fig. 5, lane 3). The haemolymph from whiteflies fed preimmune serum did not detect *E. coli* GroEL (Fig. 5, lane 2). These results indicate that anti-GroEL antibodies reached the haemolymph and retained their biological properties.

The fate of TYLCV in the haemolymph of insects fed anti-*Buchnera* GroEL antibodies was investigated. Insects were fed for 24 h with the antibodies or with preimmune serum then for 9 h on infected tomato plants showing disease symptoms. To allow virus to reach the haemolymph the insects were allowed to feed for an additional 14 h on cotton plants. Southern blot hybridization of equal amounts of haemolymph nucleic acids (Fig. 6A) showed that viral DNA was undetectable in haemolymph of insects fed anti-*Buchnera* GroEL antibodies. At the same time, viral DNA was readily detected in untreated viruliferous insects and in insects fed preimmune serum (Fig. 6B). The ~470-bp TYLCV DNA fragment amplified from viruliferous whiteflies was also amplified from the haemolymph of the insects fed preimmune serum and from the haemolymph of insects treated with anti-*Buchnera* GroEL antibodies (Fig. 6C). These results showed that the presence of the anti-*Buchnera* GroEL antibodies in the haemolymph of insects was accompanied by a dramatic reduction in the amount of TYLCV DNA, to a point where it could be detected only by PCR.

To exclude the possibility that the decrease in TYLCV transmission and in TYLCV DNA in insects fed anti-GroEL antiserum was due to degradation of viral particles by factors present in the rabbit serum, the antiserum

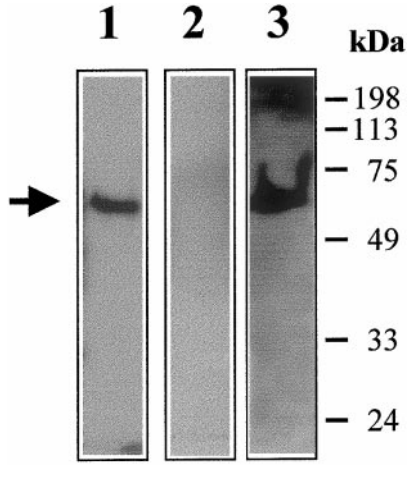

FIG. 5. Presence of biologically active anti-*Buchnera* GroEL antibodies in the haemolymph of whiteflies fed anti-*Buchnera* GroEL antiserum. *E. coli* GroEL was subjected to SDS–PAGE followed by immunodetection using (1) haemolymph of insects fed anti-*Buchnera* GroEL antiserum as a putative source of antibodies, (2) haemolymph of insects fed preimmune serum, and (3) anti-*Buchnera* GroEL antibody. The arrow points to the *E. coli* GroEL.

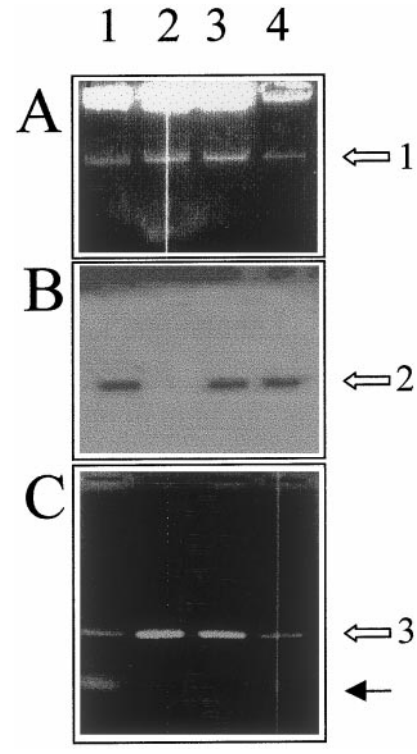

FIG. 6. Decrease of TYLCV DNA in haemolymph of whiteflies fed anti-*Buchnera* GroEL antibodies before TYLCV acquisition. DNA was extracted from the haemolymph of eight insects treated with anti-*Buchnera* GroEL antibody (lane 2), from the haemolymph of eight insects treated preimmune serum (lane 3), from the haemolymph of eight viruliferous whiteflies (lane 4), and from a single viruliferous insect (lane 1). All insects were caged with infected tomato plants for the same period of time. The DNA samples were subjected to gel electrophoresis and stained with ethidium bromide (A), blotted, and hybridized with a radiolabeled TYLCV-DNA probe (B). Viral DNA was amplified from the insect DNA samples, and the PCR products were subjected to gel electrophoresis (C). Arrows 1–3 point to the haemolymph DNA or insect total DNA, to the TYLCV genomic DNA, and to the viral DNA fragment amplified by PCR, respectively (the thin arrow indicates the position of the primers).

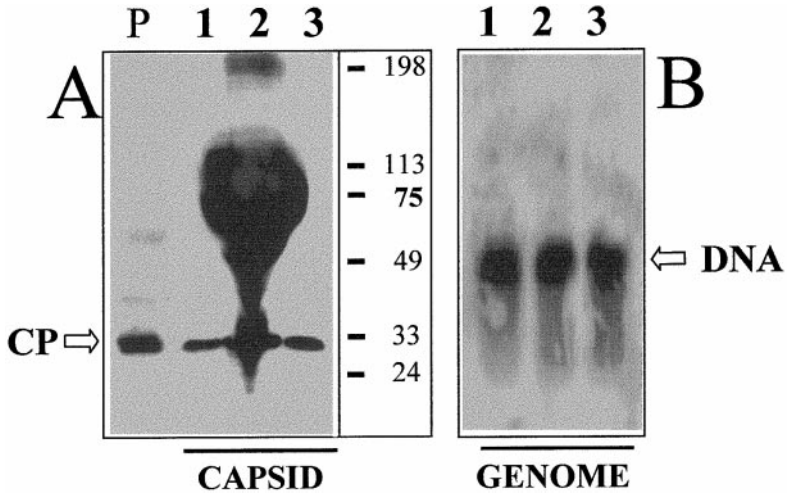

FIG. 7. Incubation with GroEL antiserum has no effect on the integrity of TYLCV capsid protein and DNA in the diet. Virions were incubated with antiserum raised against *Buchnera* GroEL (lane 1), preimmune serum (lane 2), or PBS (lane 3). An extract from an infected plant was loaded in lane P. (A) Virus protein was subjected to SDS–PAGE, immunoblotted, and reacted with an antibody against the coat protein (CP). (B) Virus DNA was subjected to agarose gel electrophoresis, Southern blotted, and hybridized with a virus-specific DNA probe. Arrows point to the virus coat protein and genomic DNA. Note: The blotch in A, lane 2, is due to the excess of protein in the preimmune serum.

raised against *M. persicae* GroEL was incubated with virions for 24 h. In parallel, virions were incubated with preimmune serum and with PBS. Analyses with specific DNA probes and antibodies showed that the effect of the anti-GroEL antibody on the integrity of the virus DNA and capsid protein was negligible, similar to the effect of the preimmune serum and to PBS (Figs. 7, A and B).

DISCUSSION

The whitefly *B. tabaci* transmits geminiviruses in a circulative manner. Similar to virus translocation in aphids (Gildow, 1987; Gray, 1997), once acquired by the whitefly during feeding, geminiviruses may be shuttled through the digestive system into the haemocoel and then to the salivary glands (Hunter *et al.,* 1998). A GroEL homologue produced by the primary endosymbionts of aphids has been shown to play a crucial role in the transmission of luteoviruses (van den Heuvel *et al.,* 1994). This protein displays high affinity to the virus capsid, and although direct association of GroEL and virus *in vivo* has not been determined, it may form a complex with virions to facilitate their passage through the hostile environment of the insect until they can be transmitted to plants. The results presented here suggest a similar involvement of a GroEL homologue from whitefly endosymbiotic bacteria in the transmission of TYLCV. The structural and biological properties of this protein are strikingly similar to those of the aphid *M. persicae*, suggesting a conserved mechanism underlying circulative transmission of viruses by their insect vector.

An antiserum raised against aphid *Buchnera* GroEL allowed us to identify a ~63 kDa protein from the whitefly *B. tabaci*. The whitefly and aphid proteins share 80% homology in the sequence of the 30 N-terminal amino acids. Electron microscopic observation of the whitefly GroEL homologue-enriched preparations revealed structures similar to those exhibited by other GroEL molecules (Filichkin *et al.,* 1997; van den Heuvel *et al.,* 1997). Collectively, these results indicate that the *B. tabaci* ~63-kDa GroEL homologue is a member of the chaperonin-60 family (Gupta, 1995).

The whitefly GroEL homologue was immunolocalized to the cytoplasm of the insect C-type endosymbionts. Two morphologically distinct C-types, which labeled equally well with the *Buchnera* GroEL antibodies (Fig. 1), have been recognized in the mycetocytes of *B. tabaci*. The predominant C-type was generally smaller (Fig. 1B) than the one resembling the aphid primary endosymbiont (Fig. 1A). Knowing that the relative frequency of the different types of microorganism within whitefly mycetocytes differs significantly between species and biotypes (Costa *et al.,* 1995), it will be of great interest to assess whether the microfauna of whiteflies affects their competence to transmit TYLCV and other begomoviruses.

The question of whether the *B. tabaci* GroEL homologue mediates the circulative transmission of TYLCV was investigated. The involvement of aphid *Buchnera* GroEL in PLRV transmission was demonstrated by feeding insects antibiotics known to inhibit protein synthesis in prokaryotes. As a consequence, the amount of GroEL in the haemolymph decreased. This decrease was correlated with a marked inhibition of virus transmission and with a reduction in the amount of virus coat protein in the haemolymph (van der Heuvel *et al.,* 1994). Similarly, we tried to reduce the amount of *B. tabaci* GroEL homo-

logue in the haemolymph by feeding insects 24 h after emergence with 75 μg/ml tetracycline in 15% sucrose before TYLCV acquisition. This treatment did not produce any reduction in the amount of haemolymph GroEL homologue and in TYLCV transmission. Therefore, we fed insects with an antiserum raised against native *Buchnera* GroEL from *M. persicae* to block the putative binding sites between GroEL and TYLCV. Functional antibodies were recovered from the insect haemolymph, demonstrating that immunoglobulins were able to move into the digestive system and to cross the gut into the haemolymph. Western blot immunodetection indicated that the concentration of antibodies in the haemolymph was ~1/1000 of that present in the diet. Similar observations were reported for other arthropods (Ben-Yakir and Schochat, 1996). The presence of the antibody was correlated with a reduction of TYLCV transmission by 83–96% and with a reduction of TYLCV DNA in the haemolymph of treated whiteflies below the threshold of detection by Southern blot hybridization. However, PCR was able to amplify viral DNA from the haemolymph of treated insects, indicating that the viral DNA did not disappear completely, a result in agreement with the residual capacity of the treated insects to transmit the virus.

In vitro binding studies indicated that the whitefly GroEL homologue has affinity for TYLCV particles. We assume that in the haemolymph of insects treated with anti-*Buchnera* GroEL antibodies, the antibody prevents binding of GroEL homologue to viral particles, leaving virions unprotected and prone to destruction by haemolymph enzymes. To be transmitted by the whitefly, TYLCV particles must associate with the GroEL homologue protein. In free-living bacteria, chaperonins have the capacity to bind proteins. They promote folding of non-native protein through an ATP-dependent process and assist in the assembly of multimeric protein complexes (Frydman and Hartl, 1994). In aphids, the capacity of GroEL to interact with luteoviruses resides in the N-terminal (amino acid residues 1–121) and C-terminal (amino acid residues 409–474) regions of the equatorial domain (Hogenhout *et al.,* 1998). These regions are highly conserved among *Buchnera* GroEL homologues, molecules that are able to bind different luteoviruses (van den Heuvel *et al.,* 1997). The high N-terminal amino acid sequence identity between *B. tabaci* GroEL and *Buchnera* GroEL indicates that at least this region of the molecule has been conserved during evolution. Future molecular studies may reveal whether the equatorial domain of *B. tabaci* GroEL also is involved in the binding of TYLCV. Luteoviruses bind *Buchnera* GroEL through the N-terminal region of the coat protein readthrough domain (van den Heuvel *et al.,* 1997). Because we found no homology between luteoviruses readthrough domain and TYLCV coat protein, we speculate that binding generally is not sequence specific but could be mediated through hydrophobic regions or specific secondary structures typically exposed by the TYLCV coat protein. The involvement of such sequences in GroEL polypeptide binding was previously described for *E. coli* (Lin *et al.,* 1995; Schmidt and Buchner, 1992).

MATERIALS AND METHODS

Virus, whiteflies, and antibodies

Bemisia tabaci of the B biotype (Cohen, 1993) was reared on cotton plants (*Gossypium hirusutum* cv Akala) grown in insect-proof wooden cages at 24–27°C, as previously described (Zeidan and Czosnek, 1991). An isolate of TYLCV from Israel (Navot *et al.,* 1991) was maintained in tomato plants (*Lycopersicon esculentum* cv. Daniella) by whitefly-mediated transmission. These plants served as source for virus acquisition by whiteflies.

Virions were purified from *Nicotiana benthamiana* leaves 4 weeks after agroinoculation of TYLCV with *Agrobacterium* At::pTY4 (Kheyr-Pour *et al.,* 1994; Navot *et al.,* 1991) according to the method developed for isolation of squash leaf curl virus (Cohen *et al.,* 1983).

Antibodies to native *Buchnera* GroEL from *Myzus persicae* were raised in rabbit as described previously (van den Heuvel *et al.,* 1997). Dr. B. D. Harrison (SCRI, Dundee, Scotland) kindly provided the monoclonal antibody SCR18 (from mouse) that recognized the TYLCV coat protein (Macintosh *et al.,* 1992).

Immunolocalization of *B. tabaci* GroEL homologue

B. tabaci adults, at 4–7 days after emergence, were fixed for 16 h in 0.1 M cacodylate buffer, pH 7.2, containing 4% (v/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde; dehydrated; and embedded (van Lent *et al.,* 1990) in LR Gold. Ultrathin sections mounted on nickel grids were labeled with 2.5 μg of anti-*Buchnera* GroEL antibody/ml of PBS for 3 h at room temperature followed by a 1.5-h exposure to goat anti-rabbit antibodies linked to gold particles (10-nm diameter). Sections were stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds, 1963) and examined using a Philips CM12 electron microscope.

Gel electrophoresis, protein blotting, and immunodetection

Whole-body homogenates of insects were prepared in Laemmli's buffer (Laemmli, 1970) (100 adult *B. tabaci* in 100 μl) and subjected to 10% SDS–PAGE. After electrophoresis, proteins were either stained with Coomassie Brilliant Blue or electroblotted at 4°C onto a Hybond-C extra membrane (Amersham) for 2 h at 110 V, using 25 mM Tris base–192 mM glycine (pH 8.3) containing 10% (v/v) methanol (transfer buffer). All subsequent steps were done at room temperature (22–25°C). The mem-

branes were blocked with 5% (w/v) skimmed milk powder in PBS containing 0.1% Tween 20 (PBS–Tween) for 1 h. GroEL and TYLCV coat protein were immunodetected as follows. Membranes were incubated for 3 h with the respective antiserum. After five 10-min washes in PBS–Tween, membranes were incubated for 3 h with horseradish peroxidase-linked anti-mouse IgG in the case of TYLCV or anti-rabbit IgG in the case of GroEL. After washes with PBS–Tween, immobilized conjugates were visualized by enhanced chemiluminescence (ECL; Amersham Life Science) followed by exposure to x-ray film.

Purification of the *B. tabaci* GroEL homologue and of *E. coli* GroEL

Adult whiteflies were homogenized in 10 ml of PBS (2 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.14 M NaCl, 2 mM KCl) containing 0.5% Triton X-100 and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was sonicated for 4 min with an Ultrasonic Processor Sonicator model W-375 (Heat System; Ultrasonics, Inc.) and centrifuged at 10,000*g* for 15 min to remove the debris. A 40% PEG-6000 solution was added to the supernatant to a final concentration of 8%. After 1.5 h on ice, the suspension was centrifuged for 20 min at 18,000*g*. The pellet was suspended in 50 mM Tris–HCl, pH 7.5, containing 35 mM KCl, 25 mM NH_4Cl, 10 mM Mg acetate, and 1 mM dithiothreitol. After 1 h on ice, the suspension was centrifuged for 15 min at 18,000*g*. The supernatant was laid onto 11 ml of a 10–50% linear sucrose gradient in the same buffer and was centrifuged in a Beckman SW41 rotor for 11 h at 37,000 rpm. The gradient was fractionated into 0.5-ml aliquots, and 20-μl samples were subjected to SDS–PAGE. The gel was electroblotted at 170 mA onto polyvinylidene difluoride (PVDF) membranes for 2 h at 4°C, using 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid containing 10% (v/v) methanol (pH 11). After staining the membrane with Coomassie Brilliant Blue, the GroEL-containing bands were excised and subjected to N-terminal amino acid sequencing. For the purification of *E. coli* GroEL, DH5α cells were grown at 37°C until an optical density of 0.6 was attained at 600 nm. The cells were then transferred to 45°C. After 16 h, the cells were pelleted, suspended in PBS, and sonicated for 4 min. Further purification was carried out as described for *B. tabaci* GroEL.

Virus overlay assay and immunodetection

Total *B. tabaci* protein extracts were subjected to 10% SDS–PAGE. After electrophoresis, gels were conditioned in transfer buffer for 1 h, and proteins were electrotransferred onto a Hybond-C extra membrane. Protein blots were incubated for 16 h with purified TYLCV particles in PBS–Tween (~50 ng viral DNA/μl) containing 2% polyvinylpyrrolidone and 0.2% BSA. The membranes were washed with PBS–Tween, and the TYLCV particles were immunodetected as described above.

Transmission of TYLCV by whiteflies fed antibodies

B. tabaci adults, at 4–7 days after emergence, were fed through membranes for 24 h on a 15% sucrose solution containing antiserum (~1 μg/μl) raised against native *Buchnera* GroEL from *M. persicae*. The insects were then fed for 9 h with a 15% sucrose solution containing the *Buchnera* GroEL antibody together with purified TYLCV particles (~0.1 μg of viral DNA/μl). Control adult whiteflies were fed for 24 h with the 15% sucrose solution containing preimmune serum (~15 μg/μl), followed by sucrose containing preimmune serum and purified virus particles (~0.1 μg of viral DNA/μl). Then, whiteflies were caged for 5 days with tomato seedlings, with one insect per plant. After 21 days, squashes of tomato leaves were hybridized with a radiolabeled TYLCV-DNA probe as described previously (Navot *et al.,* 1989), and disease symptoms were monitored thereafter.

Detection of ingested anti-*Buchnera* GroEL antibodies in insect haemolymph

Whiteflies were fed the *Buchnera* GroEL antibody or preimmune serum as described above. After 1- h starvation, the insect thorax was punctured under mineral oil, and the exuding haemolymph from 25 insects was collected in PBS–Tween containing 2% polyvinylpyrrolidone and 0.2% BSA. Protein blots containing GroEL purified from *E. coli* were incubated for 16 h with the haemolymph preparation diluted to 1 ml with the same medium. After washes with PBS–Tween, the blots were incubated for 3 h with horseradish peroxidase-linked anti-rabbit IgG. The immobilized conjugates were visualized as described above.

Analysis of TYLCV in whitefly haemolymph

Whiteflies were fed the *Buchnera* GroEL antibody or preimmune serum as described above. Subsequently, the insects were fed for 9 h on infected tomato plants followed by 14 h on cotton plants. The insect thorax was punctured under mineral oil, the haemolymph was collected with a micropipette, and DNA was extracted as follows. Haemolymph from 15 whiteflies was homogenized in 100 μl of 100 μg/ml proteinase K–0.4% SDS, incubated for 1 h at 55°C, treated with phenol/chloroform/isoamyl alcohol (25:24:1), and ethanol precipitated. The nucleic acid pellet was suspended in 80 μl of water. Total DNA purified from a single whitefly (Ghanim *et al.,* 1998) was suspended in 40 μl of water. Haemolymph and whitefly total DNA (40 μl each) were subjected to 1% agarose gel electrophoresis, blotted, and hybridized with a radiolabeled full-length TYLCV DNA probe (Navot *et al.,* 1991). Viral DNA (2 μl of haemolymph DNA or total DNA diluted 1:10 in water in 20 μl of reaction mix) was am-

plified by PCR (Ghanim *et al.,* 1998) using primers V781 and C1256 (Atzmon *et al.,* 1998).

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REFERENCES

Atzmon, G., van Hoss, H., and Czosnek, H. (1998) PCR-amplification of tomato yellow leaf curl virus (TYLCV) from squashes of plants and insect vectors: Application to the study of TYLCV acquisition and transmission. *Eur. J. Plant Pathol.* **104,** 189–194.

Baumann, P., Munson, M. A., Lai, C.-Y., Clark, M. A., Baumann, L., Moran, N. A., and Campbell, B. C. (1993). Origin and properties of bacterial endosymbionts of aphids, whiteflies, and mealybugs. *ASM News* **59,** 21–24.

Ben-Yakir, D., and Schochat, C. (1996). The fate of immunoglobulin G fed to larvae of *Ostrinia nubilalis. Entomol. Exp. Appl.* **81,** 1–5.

Briddon, R. W., Pinner, M. S., Stanley, J., and Markham, P. G. (1990). Geminivirus coat protein gene replacement alters insect specificity. *Virology* **177,** 85–94.

Buchner, P. (1965). "Endosymbiosis of Animals with Plant Microorganisms." John Wiley and Sons, New York.

Clark, M. A., Baumann, L., Munson, M. A., Baumann, P., Campbell B. C., Duffus, J. E., Osborne, L. S., and Moran, N. A. (1992). The eubacterial endosymbionts of whiteflies (Homoptera: Aleyrodidae) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. *Curr. Microbiol.* **25,** 119–123.

Cohen, S. (1993). Sweet potato whitefly biotypes and their connection with squash silver leaf. *Phytoparasitica* **21,** 174.

Cohen, S., Duffus, J. E., Larsen, R. C., Liu, H. Y., and Flock, R. A. (1983). Purification, serology, and vector relationships of squash leaf curl virus, a whitefly-transmitted geminivirus. *Phytopathology* **73,** 1669–1673.

Cohen, S., and Nitzany, F. E. (1966). Transmission and host range of the tomato yellow leaf curl virus. *Phytopathology* **56,** 1127–1131.

Costa, H. S., Westcot, D. M., Ullman, D. E., and Johnson, M. W. (1993). Ultrastructure of the endosymbionts of the whitefly, *Bemisia tabaci* and *Trialeurodes vaporariorum*. *Protoplasma* **176,** 106–115.

Costa, H. S., Westcot, D. M., Ullman, D. E., Rosell, R. C., Brown, J. K., and Johnson, M. W. (1995). Morphological variation in *Bemisia* endosymbionts. *Protoplasma* **189,** 194–202.

Czosnek, H., and Laterrot, H. (1997). A worldwide survey of tomato yellow leaf curl viruses. *Arch. Virol.* **142,** 1391–1406.

Filichkin, S. A., Brumfield, S., Filichkin, T. P., and Young, M. J. (1997). *In vitro* interactions of the aphid endosymbiont symL chaperonin with barley yellow dwarf virus. *J. Virol.* **71,** 569–577.

Frydman, J., and Hartl, F.-U. (1994). Molecular chaperone functions of hsp70 and hsp60. In "Protein Folding" (R. I. Morimoto, A. Tissieres and C. Georgopoulos, Eds.). pp. 251–283. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ghanim, M., Morin, S., Zeidan, M., and Czosnek, H. (1998). Evidence for transovarial transmission of tomato yellow leaf curl virus by its vector the whitefly *Bemisia tabaci*. *Virology* **240,** 295–303.

Gildow, F. E. (1987). Virus-membrane interactions involved in circulative transmission of luteoviruses by aphids. *Curr. Top. Vector Res.* **4,** 93–120.

Gildow, F. E., and Gray, S. M. (1993). The aphid salivary gland basal lamina as a selective barrier associated with vector-specific transmission of barley yellow dwarf luteoviruses. *Phytopathology* **83,** 1293–1302.

Gray, S. M. (1997). Plant virus proteins involved in natural vector transmission. *Trends Microbiol.* **4,** 259–264.

Gupta, R. S. (1995). Evolution of the chaperonin families (Hsp60, Hsp10 and Tep-1) of proteins and the origin of eukaryotic cells. *Mol. Microbiol.* **15,** 1–11.

Harris, K. F., Pesic-Van Esbroeck, Z., and Duffus, J. E. (1995). Morphology of the sweet potato whitefly, *Bemisia tabaci* (Homoptera, Aleyrodidae), relative to virus transmission. *Zoomorphology* **116,** 143–156.

Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., and Eilis, J. R. (1988). Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* **333,** 330–334.

Höfer, P., Bedford, I. D., Markham, P. G., Jeske, H., and Frischmuth, T. (1997). Coat protein gene replacement results in whitefly transmission of an insect nontransmissible geminivirus isolate. *Virology* **236,** 288–295.

Hogenhout, S. A., van der Wilk, F., Verbeek M., Goldbach, R. W., and van den Heuvel, J. F. J.M. (1998). Potato leafroll virus binds to the equatorial domain of the aphid endosymbiotic GroEL homologue. *J. Virol.* **72,** 358–365.

Hunter, W. B., Hiebert, E., Webb, S. E., Tsai, J. H., and Polston J. E. (1998). Location of geminiviruses in the whitefly *Bemisia tabaci* (Homoptera: Aleyrodidae). *Plant Dis.* **82,** 1147–1151.

Ishikawa, H. (1982). Host-symbiont interaction in protein synthesis in the pea aphid, *Acyrthosiphon pisum*. *Insect Biochem.* **12,** 613–622.

Ishikawa, H. (1984). Characterization of the protein species synthesized *in vivo* and *in vitro* by an aphid endosymbiont. *Insect Biochem.* **14,** 417–425.

Kheyr-Pour, A., Gronenborn, B., and Czosnek, H. (1994). Agroinoculation of tomato yellow leaf curl virus (TYLCV) overcomes the virus resistance of wild *Lycopersicon* species. *Plant Breeding* **112,** 228–233.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lin, Z., Schwartz, F. P., and Eisenstein, E. (1995). The hydrophobic nature of GroEL-substrate binding. *J. Biol. Chem.* **270,** 1011–1014.

Liu, S., Bedford, I. D., Briddon, R. W., and Markham, P. G. (1997). Efficient whitefly transmission of African cassava mosaic geminivirus requires sequences from both genomic components. *J. Gen. Virol.* **78,** 1791–1794.

Macintosh, S., Robinson, D. J., and Harrison, B. D. (1992). Detection of three whitefly-transmitted geminiviruses occurring in Europe by tests with heterologous monoclonal antibodies. *Ann. Appl. Biol.* **121,** 297–303.

Navot, N., Ber, R., and Czosnek, H. (1989). Rapid detection of tomato yellow leaf curl virus in squashes of plant and insect vectors. *Phytopathology* **79,** 562–568.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D., and Czosnek, H. (1991). Tomato yellow leaf curl virus, a whitefly-transmitted geminivirus with a single genomic component. *Virology* **185,** 151–161.

Ohtaka, C., Nakamura, H., and Ishikawa H. (1992). Structures of chaperonins from an intracellular symbiont and their functional expression in *Escherichia coli GroE* mutants. *J. Bacteriol.* **174,** 1869–1874.

Padidam, M., Beachy, R. N., and Fauquet, C. M. (1995). Classification and identification of geminiviruses using sequence comparisons. *J. Gen. Virol.* **76,** 249–263.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17,** 208–212.

Rybicki, E. P. (1994). A phylogenetic and evolutionary justification for three genera of Geminiviridae. *Arch. Virol.* **139,** 49–77.

Rubinstein, G., and Czosnek, H. (1997). Long-term association of tomato yellow leaf curl virus (TYLCV) with its whitefly vector *Bemisia tabaci*: effect on the insect transmission capacity, longevity and fecundity. *J. Gen. Virol.* **78,** 2683–2689.

Schmidt, M., and Buchner, J. (1992). Interaction of GroEL with an all-beta-protein. *J. Biol. Chem.* **267,** 16829–16833.

Unterman, B. M., Bauman, P., and McLean, D. L. (1989). Pea aphid symbiont relationships established by analysis of 16S ribosomal RNA. *J. Bacteriol.* **171,** 2970–2974.

van den Heuvel, J. F. J. M., Verbeek, M., and van der Wilk, F. (1994). Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by *Myzus persicae*. *J. Gen. Virol.* **75,** 2559–2565.

van den Heuvel, J. F. J. M., Bruyère, A., Hogenhout, S. A., Ziegler-Graff, V., Brault, V., Verbeek, M., van der Wilk, F., and Richards, K. (1997). The N-terminal region of the luteovirus readthrough domain determines virus binding to Buchnera GroEL and is essential for virus persistence in the aphid. *J. Virol.* **71,** 7258–7265.

van Lent, J. W. M., Groenen, J. T. M., Klinge-Roode, E. C., Rohrmann, G. F., Zuidema, D., and Vlak, J. M. (1990). Localization of the 34 kDa polyhedron envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. *Arch. Virol.* **111,** 103–114.

Zeidan, M., and Czosnek H. (1991). Acquisition of tomato yellow leaf curl virus by the whitefly *Bemisia tabaci*. *J. Gen. Virol.* **72,** 2607–2614.